OZONATION PROCESS TO ENHANCE BIODEGRADABILTY OF PETROLEUM WASTE BY *PSEUDOMONAS AERUGINOSA* BACTERIA IN MINERAL SALT MEDIUM

PROSES OZONASI UNTUK MENINGKATKAN KEMAMPUAN BIODEGRADASI LIMBAH MINYAK BUMI DENGAN BAKTERI *PSEUDOMONAS AERUGINOSA* DALAM MEDIA GARAM MINERAL

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ABSTRAK

Pencemaran yang diakibatkan oleh limbah minyak bumi telah membahayakan lingkungan, ditambah lagi teknologi pengolahannya masih belum efektif dan efisien. Hal ini dikarenakanlimbah minyak bumi merupakan limbah yang sulit untuk didegradasi oleh bakteri karena banyaknya kandungan senyawa ikatan kompleks di dalamnya. Karena itu, pada penelitian ini digunakan proses ozonasi sebagai *pretreatment* dengan tujuan untuk meningkatkan proses biodegradasi. Adapun variasi kondisi operasi yang dilakukan pada penelitian ini adalah dosis ozon dan nilai pH serta bakteri yang digunakan sebagai agen pendegradasi adalah bakteri *Pseudomonas aeruginosa.* Hasil penelitian yang diperoleh menunjukkan proses ozonasi dapat meningkatkan biodegradasi yang ditunjukkan dengan peningkatan persentase degradasi dan pertumbuhan populasi bakteri. Selain itu, kemampuan bakteri *P. aeruginosa* dalam mendegradasi limbah minyak bumi berbeda-beda untuk masing-masing dosis ozon dan pH. Persentase degradasi tertinggi dicapai saat dosis ozon 0,53 g/jam dan pH 7,48 dari 56,52% tanpa ozonasi menjadi 79,32% setelah inkubasi selama 7 hari.

Kata kunci: Limbah minyak bumi, bakteri *Pseudomonas aeruginosa*, ozonasi, biodegradasi, dosis ozon, pH

ABSTRACT

Contamination of petroleum waste has endangered the environment, yet its processing technology is not effective and efficient. It is becaused that by petroleum waste is difficult to be degraded by bacteria due to many complex bonding compounds containned in this oil waste. Therefore, this study used ozonation as pretreatment process with the aim to enhance the biodegradation process. The variations in operating conditions performed in this study are dose of ozone and pH. The bacteria used as a degrading agent is the Pseudomonas aeruginosa. The results show thatozonation process can the enhancebiodegradability as indicated by the increase of degradation and population growths of bacteria. In addition, the ability of the bacteria P. aeruginosa in degrading petroleum waste is varied with ozone doses and pH values. The highest percentage of degradation achieved at dose of 0.53 g ozone/h and pH 7.48 are approx from 56.52% without ozone pretreatment to 79.32% after 7 days incubation.

Keywords: *Petroleum waste, bacteria Pseudomonas aeruginosa, ozonation, biodegradability, ozone dose, pH*

I. INTRODUCTION

Petroleum (fossil fuels) is the biggest source of energy used by human. However, it also become a main source of environmental problems when it spills or as petroleum waste. The pollution emerges from oil refinery waste effluent and as a byproduct of the process of production, distribution and transportation. A number of industries have been widely developed several programs to reduce the pollution such as waste treatment unit installation, disposal facilities and incinerators (Udiharto 1999). While attempts have been made, but a large number of hydrocarbon contaminants still pollute the environment either caused by accidents, oil spills, even deliberate disposal into the environment.

The living organisms can be effected by petroleum waste that can be lethal (deadly) and sublethal (inhibits the growth, reproduction and other physiological processes). This is due to the presence of hydrocarbons contained in the oil, which has component of complex compounds and had enormous influence on the environmental pollution (Lasari 2010). Therefore, it is necessary to find a proper, fast, and effective way of handling the petroleum waste.

Previous studies have discovered microorganisms which are able to degrade hydrocarbons. One of the most tested microorganisms that have been effective in degrading hydrocarbons is *P. aeruginosa*. These bacteria are reported to grow well in the mineral salts medium and crude oil as the sole carbon source (Boshui 2012, Silva et al. 2006, Prakash & Irfan 2011). In other studies, microbiological activity of these bacteria in degrading hydrocarbons was found to increase with the use of biosurfactant. Guo-Hang Zhang reported (2005) that the potensial biodegradation of crude oil was assessed based on the development of a fermentation process with a strain *P. aeruginosa* which produced 15.4 g/L rhamnolipid. The result suggest that *P. aeruginosa* could degraded 58% of crude oil with direct or indirect of rhamnolipid (Zhang et al. 2005, Whang et al. 2010).

Bioremediation is the most widely method that applied in the processing of wastes containing organic compounds. However, this method has the disadvantage that is not effective because of the very long time required to degrade (3-4 months) while the overall efficiency is quite low (20-50%). Therefore, it requires a pretreatment step to simplify or reduce the stability of the structure of complex compounds in the petroleum waste to be more easily degraded by bacteria. The chemical oxidation process by using ozone is considered as an effective process, which has been applied for the water and wastewater treatment industry, and is particularly used to degrade organic compounds (Song et al. 2007) such as aromatic and cyclic compounds contained in the petroleum waste. Ozone is a strong oxidant (potential oxidation (E) of 2.07 V) that is very selective. It can easily reacted with aromatic compounds, however it is unreacted with many of saturated hydrocarbon compounds. The unique features of ozone application is its decomposition into OH radicals which are the strongest oxidant in water (E = 2.8 V) and non-selective. OH radicals react fast with many dissolved compounds in water. Therefore, in the application of ozone, oxidation reaction can take place directly (by ozone) and indirectly (by OH radicals) (Rodriguez 2008).

The aims of this study are to evaluate the change of the structure of compounds of petroleum waste and the efficiency enhancement of petroleum waste biodegradation by *Pseudomonas aeruginosa* after treated by ozonation process, and to observe the effect of pH on biodegradation.

II. METHODOLOGY

In this study, the petroleum waste was processed via two general steps: the ozonation as pretreatment process and biodegradation process by *P.aeruginosa*. Ozonation process is expected to reduce complex bond compounds composition of samples, as a result biodegradation process occure more easily. The medium used for bacterial growth was mineral salt medium, *i.e* a mixture of distilled water enriched with nutrients mineral salts. In addition, the effect of pH on biodegradation was also observed. The effect of the the pretreatment step by ozonation is indicated by changes in the characteristics of the sample, the growth of bacteria and the percentage of biodegradation. Sample characteristic was associated with the changes in the composition of hydrocarbon compounds, before and after ozonation as well as after biodegradation, which will be analyzed by GC-MS method. The growth of bacteria was evaluated by the change in the number of bacteria which was calculated by TPC (Total Place Count) method. The

percentage of biodegradation was indicated by the difference of oil and grease content before and after pretreatment and after biodegradation processes which was analyzed by the gravimetric method refer to ASTM D 4281-95.

A. Preparation of Bacterial Cultures

The bacteria used was pure culture of *P. aeruginosa* from BLCC (Biotechnology Lemigas Culture Collection). The culture was activated in NB medium (Nutrient Broth) for 24 hours at room temperature. After 2 times activation period, bacterial culture was gradually adjusted to 100 mL of treatment medium in the form of mineral salts medium and 0.1% (v/v) crude oil. The content of the mineral salts medium is (gL^{-1}): 0.1 g NH_4NO_3, 0.1 g K_2HPO_4, 0.05 g $MgSO_4.7H_2O$, 0.001 g $FeCl_3$, 0.01 g KCl, and 0.001 g $CaCl_2$ dissolved in 1 L distilled water. Cultures were incubated for 72 hours at room temperature at 120 rpm. After 3 times of adaptation period, 10% (v/v) containing ± 10^7sel/mL culture, ready for use in the process of biodegradation of petroleum waste.

B. Petroleum Waste Sample Preparation

Petroleum waste used as the sample was the mixture of crude oil from the refinery PPT Migas Cepu, Central Java, and Mineral salts medium with crude oil concentration of 3% (30,000 ppm) was prepared by dissolving 5 g of crude oil into 150 ml mineral salt medium. All samples including the tools were sterilized in an autoclave at 121°C for 15 minutes before ozonation. For acidic pH variation, H_2SO_4 5 N solution was used, while for alkaline pH variation, NaOH 5 N solution was used.

C. Ozonation Step

The process was carried out in batch system for 30 minutes with variations of ozone dose and pH values. The equipment used was ozonator and batch reactor. Ozone was produced in-situ from the ozonator. Compressed air was passed to the ozonator with certain flowrate to produced ozon-air mixture with certain ozone dose. The experimental set-up was arranged according to Figure 1.

150 mL petroleum waste sample which has been prepared previously incorporated into the reactor than ozone was bubbled into the reactor for 30 minutes. The variation made was feed air flow rate was arranged between 100 L/h to 400 L/h and pH values starting from 5 to 8 (using a strong acid and alkaline solution H_2SO_4 and NaOH).

D. Biodegradation Step

Petroleum wastes samples was treated by ozonation were added by bacteria which had been adapted from mineral salts. These samples were incubated for 7 days at room temperature and than shaked at 120 rpm. The performance of biodegradation was evaluated based on the change of oil and grease content, pH, and hydrocarbon composition, and the growth of the bacteria. The observations were made on day 0 and day 7.

E. Oil and Grease Content Analysis

The acidified water samples were extracted with n-hexane solvent in a separatory funnel. Then the solvent was evaporated by heating on a hot water bath. The sample obtainned from the extraction was placed in an oven for 1-2 days to evaporate the remaining solvent. The dried sample than put in desiccator for 1 hour, remove and weigh immediately.

F. Hydrocarbon Composition Analysis

Hydrocarbon composition was analyzed by GCMS method. Sample of petroleum liquid was introduced into a gas chromatograph equipped with an open tubular column. Helium carrier gas transported the vaporized sample through the column, in which separator process occures. The individual sampels is then detected with FID. Each eluting component was identified by comparing its retention time to that established by analyzing reference standards. The concentration of each component in mass % was determined by normalization of peak

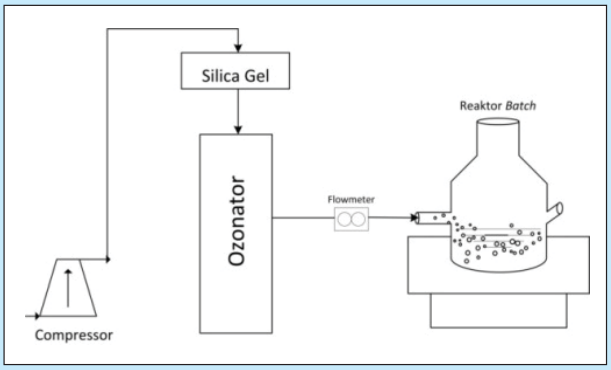


Figure 1
Experimental set-up

areas after correction with detector response factor.

III. RESULTS AND DISCUSSION

A. Determination of Oil and Grease Content

Oil and grease is a parameter used to determine the percentage of biodegradation in the process of biodegradation by bacteria. Determination of oil and grease wsa performed by gravimetric method on samples with variations of ozone dose and pH. Ozone dose was varied by changing the flow rate of feed air into ozonator. Compressed air was passed to the ozonator with certain flowrate to produced ozon-air mixture with certain ozone dose. The results of the calculation of ozone dose on each variation of air flow rates used are listed in Table 1 below:

Table 1
Ozone dose calculation results

Air flow rate(L/h)	Ozone dose (g/h)
100	0.35
200	0.41
300	0.46
400	0.53

1. Percentage of Degradation with Ozone Dose Variations

Biodegradation process from day 0 to day 7shows a decrease in oil and grease content on the ohter hand an increase in the percentage of degradation as the ozone dose increased. Figure 2 shows a decrease of oil and grease content after ozonation for 30 minutes and after biodegradation with an incubation period of 7 days while Figure 3 shows the percentage of degradation in a variety of ozone doses used. The control here is an initial sample without ozonation pretreatment.

Figure 2 and Figure 3, shows that the highest decline in oil and grease achieved at the highest ozone dose of 0.53 g/h, from 32,660 ppm on day 0 to 5,472 ppm on day 7 with the biodegradation rate of 79.32%. From these figures we can also concluded that the influence of ozonation determine the final condition after biodegradation. This can be seen from the condition of the sample after ozonation for each variation of ozone dose. Sample with the highest decline of oil and grease content after ozonation also provide the highest decline of oil and grease content

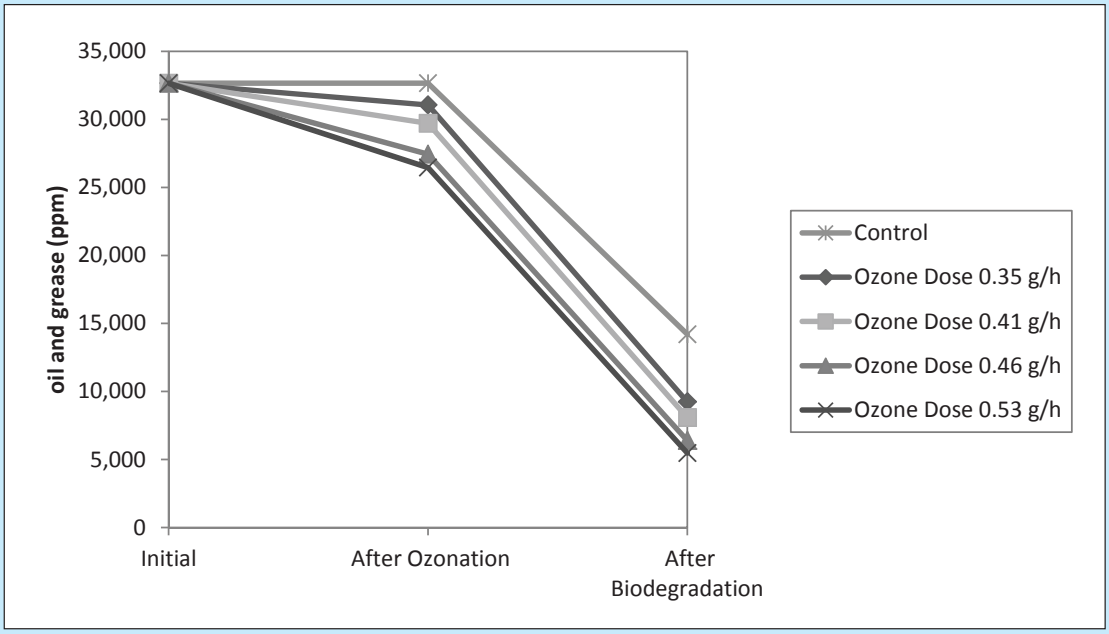


Figure 2
Oil and grease content for ozone dose variation

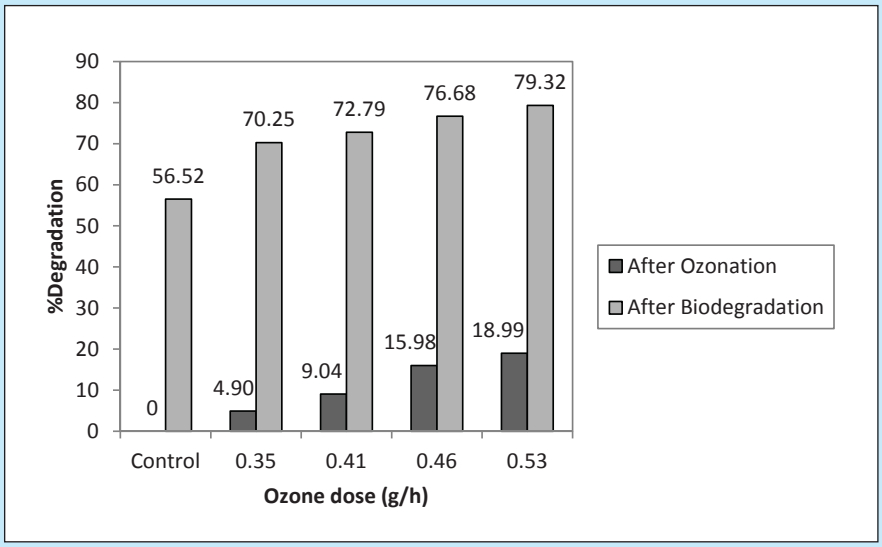


Figure 3
Percentage of degradation for ozone dose variation

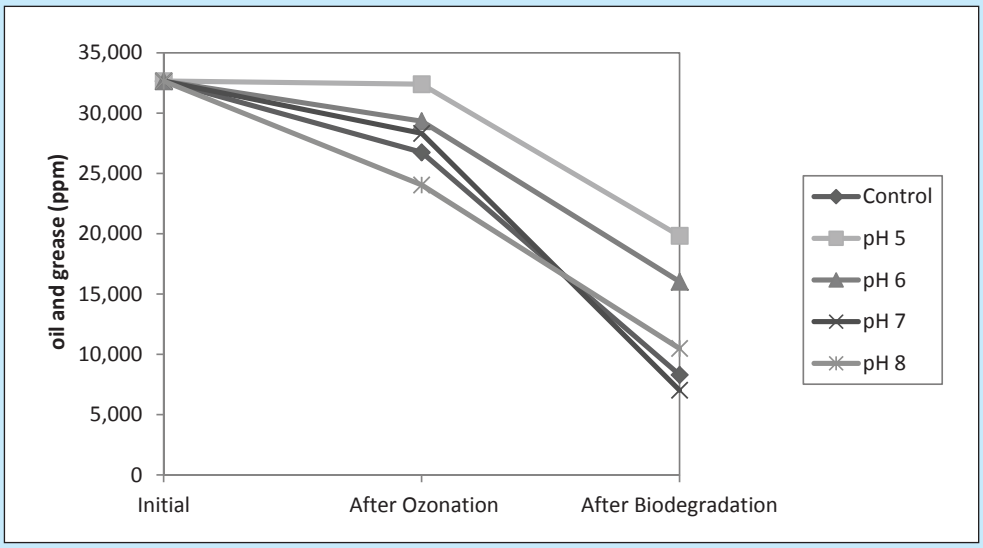


Figure 4
Oil and grease content for pH variation

after biodegradation as well as its percentage of degradation. Thus, from the initial conditions after ozonation we can predict the samples give the best result (highest degradation).

This condition is caused by an increase in ozone dose given during pretreatment. The more ozone dose is given the more OH radicals generated from decomposition of ozone. These two oxidants (ozone and OH radicals) reacted with complex hydrocarbon compounds (aromatics, paraffinic and naphtenic) contained in the petroleum waste generating simpler hydrocarbon molecules (such as aldehydes and alcohols) as can be seen in from GC-MS analysis result which is discussed in other part of this paper.

The more simpler hydrocarbon molecules further leads to increase growth of the bacterial population in

the medium that will ultimately increase the activity of bacteria in degrading petroleum waste. Microbes will degrade hydrocarbons as a carbon source to produce energy for its survival and will produce a gas, organic acids, and biomass (Karwati 2009).

2. Percentage of degradation for pH variation

Figure 4 that shows the oil and grease content after ozonation with a fixed dose of ozone that is 0.53 g / h for 30 minutes and after biodegradation for 7 days with pH variation. In addition the ability of the bacteria *P. aeruginosa* in degrading petroleum waste is varied with pH value. While the percentage of degradation for each pH value is seen in Figure 5. Based on these two figures, it is exhibitted the decline in oil and grease content quite high occurred at pH 7 and pH control (initial sample pH 7.48) with successive degradation rate of 75.21% and 74.57%.

In contrast to the results of ozone dose variation, in which the initial condition after ozonation determine the final condition after biodegradation, not so in this pH variation. Initial conditions after ozonation provide different decline of oil and grease content though given the same dose of ozone, which is 0.53 g / h for 30 minutes. This is because the pH value can affect the action of ozone on organic matter, which can be either direct or indirect ozonation.

Figure 4 and Figure 5 also show that the highest decline of oil and grease content as well as the highest percentage of degradation after ozonation present in the highest pH used in this study (pH 8). This is because under alkaline conditions, the solution will be dominated by the OH^- ions, which will initiate ozone to decompose into OH radicals. The higher the pH value, the more ozone are converted into OH radicals. The OH radical is a more powerful oxidizing agent than ozone and has a low selectivity, so that more number of OH radicals in the reaction system, the more organic compounds are attacked/oxidized by these radicals (Pera-Tirus 2004).

pH gives difference effect on biodegradation compared to ozonation process. The highest decline of oil and grease content at pH 8 in ozonation process is not followed by highest decline of oil and grease content at the same pH for biodegradation process. Theoretically, higher pH (pH 8 in this research) gives higher number of OH radicals, which further convert more complex hydrocarbon compounds into simpler ones. However, bacterial growth in the biodegradation process is highly affected by the environment conditions, such as nutrition, pH, dissolved oxygen and temperature. The optimum pH for the growth of *P.aeruginosa* bacteria is 6.6 -7.0 (Todar 2012). This range of pH is about the same with the pH of control sample (pH 7.48).

Thus, although the composition of the compound that difficult for bacteria to degrade after ozonation successfully reduced, the enhancement

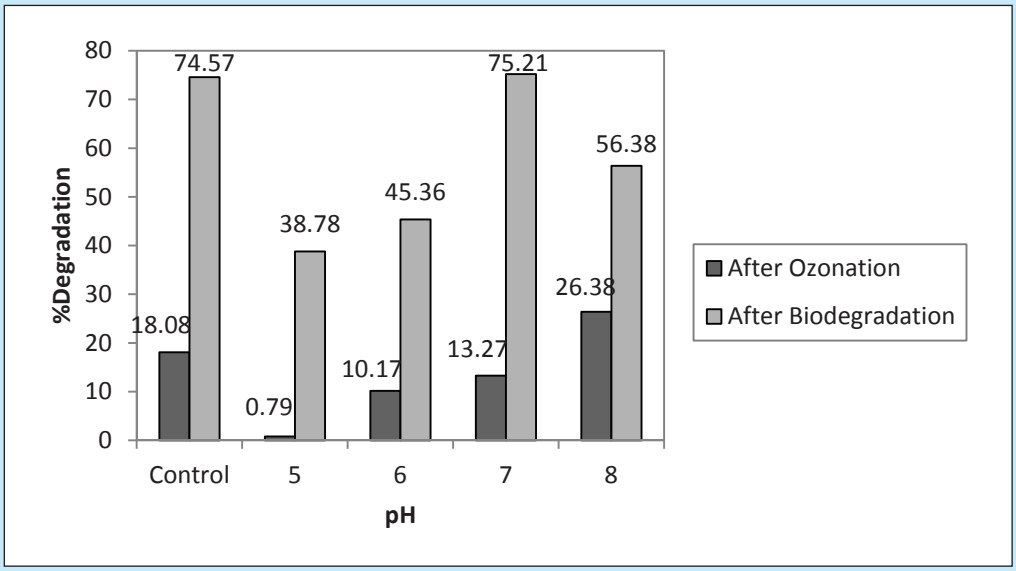
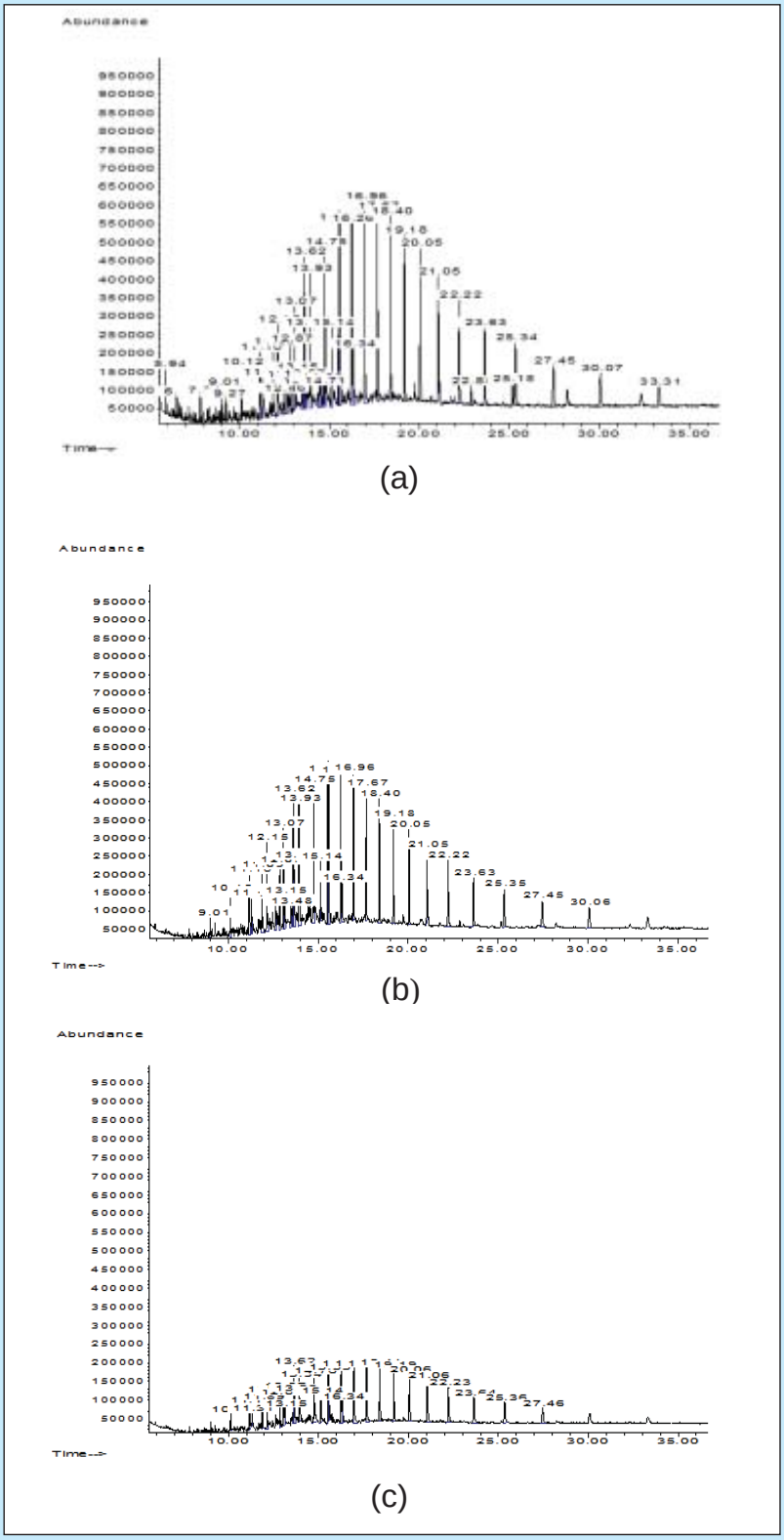


Figure 5
Percentage of degradation for pH variation



(a) chromatogram ofcrude oil Cepu,
(b) chromatogram of sample after ozonation with ozone dose 0.53 g/h,
(c) chromatogram of sample after biodegradation 7 days.

Figure 6. GCMSChromatogram Profiles

of biodegradation becomes insignificant if the core agents in the biodegradation i.e bacteria *P. aeruginosa* is difficult to grow in medium, due to the condition of the pH that is not suitable for its growth. This also explains why the percentage of degradation on value of pH 5 and 6 is not too high.

pH values associated with the activation of the enzyme decomposer petroleum existing in bacteria. Biodegraded by microbes may occur in the presence of bacterial growth and enzyme activity possessed by microorganisms. Through these enzymatic process, bacteria cantransform hydrocarbon substance into a more modest compound (Atlas & Bartha 1998). At the optimal pH value, these enzymes will be more activated as indicated by the higher percentage of biodegradation (pH 7).

B. Petroleum Component Analysis

Changes in the structure of petroleum before and after ozonation phase and biodegradation can be determined by using GCMS instrument. This study only took 3 samples for GCMS analysis, namely crude oil Cepu,sample after ozonation with ozone dose 0.53 g/h, and the samples after biodegradation. GCMS analysis results can be observed from the following chromatogram profiles (Figure 6).

From the profile of the chromatogram,we obtained the content of the compounds found in petroleum waste in initial sample, after ozonation, and after biodegradation. This study only focused on three compounds which are usually difficult to be degraded by bacteria, namely paraffinic compounds (both short-and long-chain compounds), naftenic compounds (cycloalkanes), and aromatics. Figure 7 shows the decline in abundance (concentration) of paraffinic compounds, naftenics, and aromatics in the sample at the beginning, after ozonation for half an hour, and after biodegradation for 7 days.

Figure 7, Exhibit can be observed that the composition of the paraffinic compounds, naftenics and aromatics always declining until the biodegradation process. This result is synergic with the previous results, that the highest decline of oil and grease as well as the percentage of biodegradation occured in this ozone dose levels. It clearly shows thatthe ozonation process can enhance the biodegradation and also *P. aeruginosa* bacteria were able to degrade petroleum used as indicated

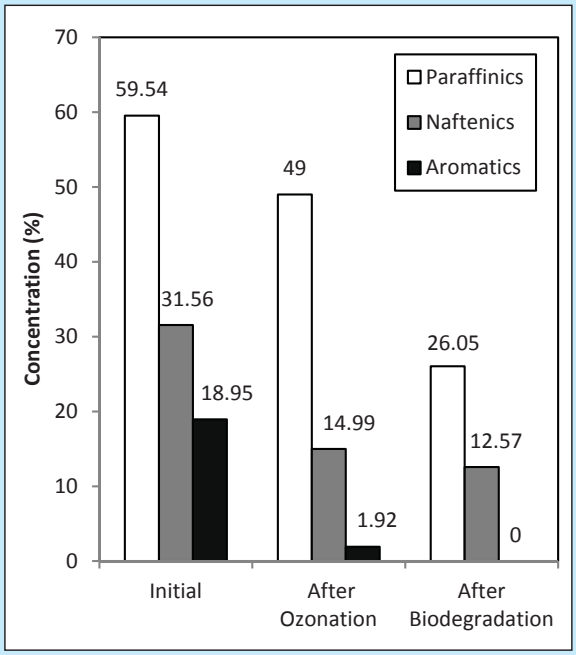


Figure 7
Decline in the concentration of paraffinics, naftenics, and aromatics compound in the petroleum waste samples ozone dose 0.53 g/h

by changes in the structure of petroleum into more simple compound.

C. Observation of Bacterial Growth

The observation of the growth rate of *P. aeruginosa* in the incubation medium was conducted in the biodegradation step. This observation was carried out for the variation of ozone dose and pH values. These two variations were conducted since the first step of the research, i.e. ozonation process as pretreatment for biodegradation. Based on the characteristic of ozone which is easily decomposed to oxygen, it can be confirmed that ozone was not exist in the solution when the biodegradation step was carried out. The bacterial growth was performed by counting the number of the bacteria on day 0 and day 7 and calculate daily growth coefficient (μ). The growth of the bacterial population to ozone dose variation can be seen in Table 2 below.

Meanwhile, Table 3 shows the population growth of *P. aeruginosa* were incubated for 7 days at the Shaker Table at a variety of pH values.

The increase of population growth in the petroleum containing medium indicated that the bacteria *P. aeruginosa* is active in the petroleum

Table 2
Population of P. Aeruginosa bacteria for ozone dose variation

Ozone Dose (g/h)	CFU/mL		μ
	Day-0	Day-7	
Control	2.0×10^9	5.0×10^9	0.13
0.35	3.0×10^9	1.2×10^{10}	0.20
0.41	2.0×10^9	1.0×10^{10}	0.23
0.46	4.0×10^9	2.5×10^{10}	0.26
0.53	3.0×10^9	2.3×10^{10}	0.29

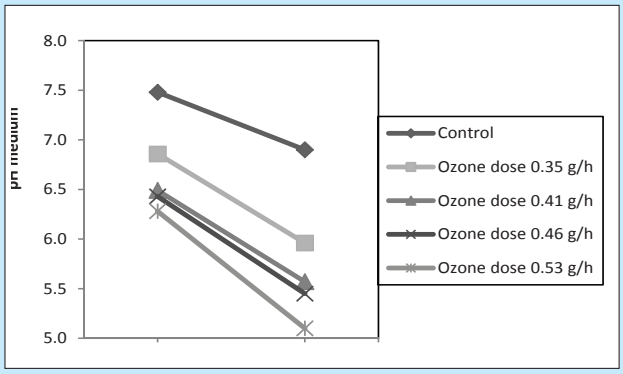


Figure 8
pH medium after 7 days incubation for ozone dose variation

Table 3
Population of P. aeruginosa bacteria for pH variation

pH	CFU/mL		μ
	Day-0	Day-7	
Control	3.0×10^9	8.0×10^9	0.14
5	5.0×10^9	7.0×10^9	0.05
6	3.0×10^9	6.0×10^9	0.10
7	3.0×10^9	1.10×10^{10}	0.19
8	4.0×10^9	9.0×10^9	0.12

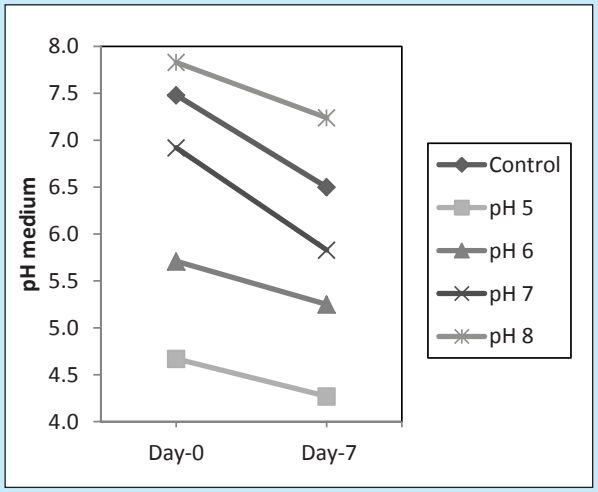


Figure 9
pH medium after 7 days incubation for pH variation

environment and have the ability to degrade petroleum hydrocarbons.

In Table 2 it can be seen that the bacteria *P. aeruginosa* has a growth rate that is likely to rise with the increased of ozone dose in the ozonation pretreatment process, where the highest growth rate was achieved at the ozone dose of 0.53 g/h. The increase of bacterial growth in the biodegradation process with increasing dose of ozone in the ozonation pretreatment process proved the ability of ozone (and the generated OH radicals) to convert complex hydrocarbon compounds, which are difficult to be degraded by bacteria such naftenic and aromatic compounds, into simpler compounds that is easier to degrade. This is because ozone is very selective and can easily attack naftenic and aromatic compounds and convert them into paraffinic compounds (straight chain) that are more soluble in water and diffuse into the cell membranes of bacteria (Atlas & Bartha 1998). According to Udiharto, and active bacteria potentially degrade petroleum will show high growth rates in medium containing petroleum than the medium that not contain petroleum (Udiharto 1999).

Meanwhile, for the growth of bacteria on the variation of pH values as shown in Table 3, it can be concluded that the pH value of 7 is an optimum value for the growth of *P. aeruginosa*.

Generally, bacteria have optimum growth at neutral pH (pH 7), including *P. aeruginosa*. As mentioned previously, the pH is closely associated with the activity of the enzyme in bacteria. The ability to degrade petroleum associated with the presence of hydrocarbon decomposer enzymes in bacteria that allows the bacteria to grow on pentroleum environment (Atlas & Bartha 1998). Therefore, at pH 7 these enzymes work optimally and make a higher bacterial growth.

D. Observation of pH medium

One of the parameters that proved the occurrence of biodegradation by bacteria is the change in pH of the medium. pH changes that occur in the treated bacteria medium showed the activity in the hydrocarbon remodel.

Figure 8 top shows the results of the observations of pH medium after ozonation and 7 days biodegradation for ozone dose variation. The highest decline occurred in pH medium treatment at a dose of 0.53 g ozone / h, *i.e* from pH of 6.28 to 5.10 or a decrease of 1.18 at the end of incubation.

While the pH medium after ozonation and biodegradation for pH variation shown in Figure 9. These figures shows that for the variation of pH, pH decline was the highest in the treatment medium with pH 7, *i.e* from 6.95 to 5.83 or a pH decrease of 1.09.

This decrease in pH medium occurred due to the activity of bacteria that form acid metabolites. Biodegradation of alkanes found in petroleum will form the alcohol and then into fatty acids. Fatty acid alkane degradation will further oxidized to form acetic acid and propionic acid, which can lower the pH value of the medium **(R & B.R. Atlas 1998)**.

The magnitude of decrease in pH varies and depends on the percentage of degradation and degrading bacteria. Increasing hydrocarbon degrading microbial activity will also increase the amount of organic acids produced and the greater the resulting decrease in pH value **(R & B.R. Atlas 1998)**.

IV. CONCLUSIONS

Based on the research that has been done, it can be concluded that the ozonation of petroleum waste can enhance biodegradability as indicated by the increase in the percentage of biodegradation and also the ability of the bacteria Pseudomonas aeruginosa in degrading petroleum waste vary each pH value. In addition, the biodegradation process produces organic acid compounds that can lower the pH value and the highest percentage of biodegradation for ozone dose variation obtained 79.32% achieved at doses of 0.53 g ozone/h, while for the variation of pH value obtained 75.21% achieved at pH 7.

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